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The Role of *L. monocytogenes* Serotype 4b *gtc*A in Gastrointestinal Listeriosis in A/J Mice

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Abstract

Serotype 4b strains of *Listeria monocytogenes* have been responsible for most large outbreaks of listeriosis. In *L. monocytogenes* serotype 4b, *gtcA* and *gltA* have been implicated in serotype-specific glycosylation of the teichoic acid of the cell wall with galactose and glucose. In this study, we investigated the impact of mutations in *gltA* (resulting in absence of glucose on teichoic acid) and *gtcA* (resulting in absence of galactose, and markedly reduced glucose on teichoic acid) on virulence following intragastric infection of anesthetized A/J mice. The *gltA* mutant was not impaired in virulence in this model. In contrast, testing of *gtcA* mutants constructed in two different strains showed that the mutants were recovered in lower numbers than their respective parent strains from the spleen, liver, ceca, and gall bladders of intragastrically inoculated mice. Genetic complementation of the *gtcA* mutation partially restored gastrointestinal virulence. When mice were inoculated intravenously, the *gtcA* mutants were also recovered in lower numbers from the liver (for both mutant strains) and the spleen (for one mutant strain) than their respective parental strains. The mutants were also evaluated for invasion and intracellular multiplication in the Caco-2 human intestinal epithelial cell line. Inactivation of *gltA* did not affect invasion or intracellular growth of the bacteria. In contrast, *gtcA* mutants showed decreased invasion, but normal multiplication in Caco-2 cells. Overall, these data demonstrate a role for *gtcA* in the pathogenesis of gastrointestinal listeriosis in mice, and suggest that diminished ability of *gtcA* mutants to invade intestinal epithelial cells may be partly responsible for decreased gastrointestinal virulence.

Introduction

Listeria monocytogenes is an important foodborne pathogen causing an estimated 2500 cases and up to 500 deaths per year in the United States. Although 13 serotypes are recognized, the majority of clinical cases of listeriosis involve strains of just three serotypes, specifically 1/2a, 1/2b, and 4b (Notermans *et al.*, 1998; Kathariou, 2002; Painter and Slutsker, 2007). In serotype designations for *L. monocytogenes*, the numerical portion (i.e., 1/2, 3, 4) is based on serotype-specific glycosylation of the teichoic acid of the cell wall, whereas the remaining letter-based designation (i.e., a, b, c) corresponds to flagellin-specific antigens. A substantial fraction of sporadic listeriosis and most of the large outbreaks of invasive listeriosis

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in the United States and Europe have involved strains of serotype 4b (Linnan *et al*., 1998; Notermans *et al*., 1998; Kathariou, 2002; Graves *et al*., 2005; Mead *et al*., 2005; Olsen *et al*., 2005; Gottlieb *et al*., 2006). However, the reason for the high prevalence of strains of this serotype in human foodborne illness remains poorly understood. Comparative genomic analysis reveals numerous differences between *L. monocytogenes* strains of different serotypes (Doumith *et al*., 2004; Nelson *et al*., 2004). Strains of the serotype 4b complex (serotype 4b and the closely related serotypes 4d and 4e) are unique among *L. monocytogenes* in harboring *N*-acetylglucosamine as an integral component of the teichoic acid backbone. In addition, the *N*-acetylglucosamine is decorated by glucose and galactose (Uchikawa *et al*., 1986).

Earlier studies showed that placement of glucose on teichoic acid of serotype 4b *L. monocytogenes* required the presence of *gltA*, harbored by *L. monocytogenes* of the serotype 4b complex (serotype 4b and the closely related serotypes 4d and 4e) but not other serotypes (Lei *et al*., 2001). Inactivation of another gene, *gtcA*, resulted in loss of galactose and markedly reduced amounts of glucose on the teichoic acid (Promadej *et al*., 1999). Inactivation of *gtcA* also rendered the bacteria resistant to infection by both serotype-4b specific and *Listeria* genus-specific phages (Cheng *et al*., 2008). Although *gtcA* was originally thought to be unique to serogroup 4 (Promadej *et al*., 1999), subsequent studies revealed the presence of a homologue (82% nucleotide sequence identity) in strains of serotype 1/2a and 1/2b (Autret *et al*., 2001; Glaser *et al*., 2001; Nelson *et al*., 2004; Broad Institute *L. monocytogenes* genomic sequence database, accessed at http://www.broad.mit.edu/annotation/genome/listeria_group/MultiHome.html). Signature tag mutagenesis revealed that mutants in *gtcA* of the serotype 1/2a strain EGD-e were impaired in their ability to invade the brain in a murine infection model (Autret *et al*., 2001). However, the role of *gtcA* or *gltA* in the pathogenesis of serotype 4b strains has not been described, and no information has been available on the potential involvement of these genes in the pathogenesis of gastrointestinal listeriosis.

Animal models for oral or intragastric (IG) infection with *L. monocytogenes* present a number of challenges. Mice do not express a functional receptor (i.e., human sequence E-cadherin) for the invasion-associated protein internalin A. Oral infection of transgenic mice expressing human E-cadherin and guinea pigs, which naturally express a functional receptor for internalin A (i.e., human sequence E-cadherin), have been proposed as models that simulate invasion of intestinal epithelial cells and the subsequent systemic infection of human listeriosis (Lecuit *et al*, 2001; Cabanes *et al*., 2002; Dussurget *et al*., 2004). However, these models also have limitations. For example, very high numbers of bacteria are required for infection of guinea pigs, and the human E-cadherin transgenic mice are not generally available to investigators at this time. On the other hand, despite the absence of a functional internalin A receptor in mice, IG infections have been employed successfully to evaluate relative virulence of different *L. monocytogenes* strains, assess relative outcomes of IG vs. intraperitoneal routes of infection, and compare the impact of growth matrices of the bacteria (e.g., growth in milk) on virulence (Pine *et al*., 1990). More recently, we described a *L. monocytogenes* infection model using anesthetized A/J mice (Czuprynski *et al*., 2003a,b). Mice so infected developed a systemic infection as evaluated by histopathology and recovery of *L. monocytogenes* from the blood, spleen, liver, and gall bladder. In addition, colonization of the cecum was observed (Czuprynski *et al*., 2003a; Faith *et al*., 2005, 2007). In the present study, *gltA* and *gtcA* mutants of *L. monocytogenes* serotype 4b were evaluated using this model to investigate the possible role of these genes in the pathogenesis of gastrointestinal listeriosis involving bacteria of serotype 4b.

Materials and Methods

Preparation of L. monocytogenes

The *gtcA* and *gltA* mutants employed here have been described previously (Promadej *et al*., 1999; Lei *et al*., 2001). Mutants M44 and 27E8 were derived from the serotype 4b strains 4b1 (a streptomycin-resistant derivative of the sporadic clinical isolate NCTC 11257) and 2381L (a streptomycin-resistant derivative of strain F2381, implicated in the 1985 California outbreak), respectively. Both mutants harbored a

single insertion of Tn*916*ΔE in *gtcA* (Promadej *et al*., 1999). M44pPL2-gtcA was a genetically complemented derivative of M44 in which wild-type *gtcA* along with a ca. 300 nt upstream region was integrated in $tRNA^{arg}$. The control for the complemented strain was M44pPL2, harboring the vector pPL2 alone in the same $tRNA^{arg}$ integration site. M44pPL2-gtcA and M44pPL2 were constructed using the integration vector pPL2 (Lauer *et al*., 2002) and were previously described (Cheng *et al*., 2008). Mutant XL7 was derived from 4b1 and harbored a single Tn*916*ΔE insertion in *gltA* (Lei *et al*., 2001). Bacteria were routinely grown at 37°C in brain heart infusion (BHI) broth (BD, Sparks, MD) or on tryptic soy agar supplemented with 5% sheep blood (SBA, BBL). We detected no substantial differences in the growth rates of the mutant and wild-type parent strains as evaluated using a Bioscreen instrument (Growth Curves USA, Piscataway, NJ). For long-term preservation of the strains we used *Listeria*-coated beads (Copan Diagnostics Inc., Corona, CA) following the instructions of the manufacturer. To prepare *L. monocytogenes* cells for use in this study, a single *Listeria*-coated bead was inoculated into 50 mL of BHI broth in a 250-mL Erlenmeyer flask. The culture was incubated for 20 hours with shaking at 37°C until stationary phase growth was reached. The cells were recovered by centrifugation and resuspended in an equal volume of phosphate-buffered saline (PBS). The optical density of the bacterial suspension was read with a spectrophotometer, and the colony-forming units (CFU) were extrapolated from a standard curve. Appropriate dilutions of the bacterial suspension were made in sterile PBS to achieve the desired bacterial concentration, which was verified in each experiment by plate counts on SBA.

Mouse inoculation experiments

The *gltA* and *gtcA* transposon mutants and their respective parental strains were assessed for virulence in a previously described model of gastrointestinal listeriosis, using the susceptible A/J mouse strain (Czuprynski *et al*., 2003a). Female inbred A/J mice (Harlan Sprague-Dawley, Indianapolis, IN) were obtained at 5–6 weeks of age and housed under micro-isolator caps at the School of Veterinary Medicine animal care facility at the University of Wisconsin, Madison. Mice were acclimated for at least 1 week in this facility (until their average weight reached 15 g), before being used in an experiment. Mice received food and water *ad libitum* until 5 hours prior to IG inoculation, at which time food was removed from the cage. This was done to minimize the possibility that food in the stomach might prevent delivery of the listerial inoculum and lead to aspiration into the lungs. Mice were anesthetized by IP injection with sodium pentobarbital (0.75 mg per 25 g body weight) (Abbott, Abbott Park, IL) as described previously (Czuprynski *et al*., 2003b). Once sedation occurred, the listerial inoculum was introduced (in a total volume of 0.2 mL) via a 1.5-inch 24-gauge stainless steel feeding needle attached to a 1-mL syringe. In some experiments, mice were inoculated intravenously by placing unanesthetized mice into a plastic restrainer, and injecting the inoculum of *L. monocytogenes* (in 0.2 mL) into a lateral tail vein using a 27-gauge needle. At the indicated time points following inoculation, the mice were euthanized by asphyxiation with CO_2, followed by exsanguination. Blood was collected into a syringe containing 0.1 mL sodium citrate (4%) as anticoagulant. The blood was then serially diluted in sterile saline and plated in duplicate (0.1 mL) on SBA plates that were incubated for 48 hours at 37°C. The abdominal cavity was aseptically opened and portions of the spleen, liver, gall bladder, and cecum were removed, weighed in sterile weigh boats, and placed in sterile tissue grinders that contained 1 mL cold sterile saline (0.85%). The tissues were homogenized, diluted in sterile saline, and plated in duplicate onto SBA (for spleen, liver, blood, gall bladder) or modified Oxoid agar plates (for ceca). The plates were allowed to dry and then incubated at 37°C for 48 hours. The colonies were counted and the data expressed as the mean ± SEM $\log_{10}$ CFU of *L. monocytogenes* per gram of tissue (wet weight) for spleen, liver, and cecum and per organ for the gall bladder. The limit of detection was 1.0 $\log_{10}$ CFU; tissues that did not yield any colonies were assigned a value of 0.95 $\log_{10}$CFU for calculation of the mean ± SEM of that treatment group. This study was approved by the

University of Wisconsin-Madison School of Veterinary Medicine Institutional Animal Care and Use Committee.

Caco-2 invasion assay

The ability of *L. monocytogenes* to invade and multiply within the human colonic adenocarcinoma cell line Caco-2 (ATCC HTB37, Rockville, MD) was determined as described previously (Faith *et al.*, 2005). To assess bacterial attachment, Caco-2 cells were grown and allowed to fully differentiate and form polarized monolayers over an 18- to 21-day incubation period on glass coverslips in 24-well tissue culture plates. The tissue culture medium was aspirated from each well and replaced with 1 mL of medium containing 1×10^7 CFU of *L. monocytogenes*. Following a 10-minute incubation at 37°C the medium was removed and the monolayers washed five times with warm (37°C) Hanks' Balanced Salt Solution (HBSS). The coverslips were then removed, placed in a new tissue culture plate and treated with 1% Triton-X-100 (Acros Organics, Morris Plains, NJ) in PBS to lyse the monolayers. To assess invasion, individual wells of 24-well cell culture plates (Becton Dickinson, Franklin Lakes, NJ) were seeded with Caco-2 cells at a density of 60,000 cells/well, and the cells were allowed to fully differentiate and form polarized monolayers over an 18- to 21-day incubation period. The monolayers were incubated with 1×10^7 CFU of *L. monocytogenes* for 1 hour, washed as described above, and then incubated an additional 2.5 hours in HBSS with 20 μg/mL gentamicin (Sigma, St. Louis, MO) to kill any extracellular listeriae. The cells were then washed five times with warm HBSS and lysed using 1% Triton-X-100 (Acros Organics) in PBS. Serial dilutions of the cell lysates (for both attachment and invasion assays) were plated in duplicate on SBA plates and incubated at 37°C. The colonies were counted and the data expressed as the mean ± SEM $\log_{10}$ CFU per well (nine wells per treatment group). To assess intracellular growth, the infected monolayers were incubated for 24 hours in medium with 20 μg/mL gentamicin and then the monolayers were washed and lysed, and the lysates were plated on SBA to determine CFU, as indicated above.

Statistical analysis

Data were analyzed with an analysis of variance using GraphPad Prism version 4.0 (GraphPad Software, Inc., San Diego, CA). If a significant F value was obtained ($p < 0.05$), the Tukey–Kramer or Mann–Whitney test was performed to determine whether the means of treatment groups differed from those of controls or one another. Statistical significance for all comparisons was set at $p < 0.05$.

Results

Virulence of L. monocytogenes *serotype 4b in intragastrically inoculated A/J mice requires* gtcA, *but not* gltA

We first investigated the ability of the *gltA* mutant (XL7) of *L. monocytogenes* to cause gastrointestinal listeriosis following IG inoculation of anesthetized A/J mice. No significant differences were noted between the *gltA* mutant and its parental strain in terms of CFU of *L. monocytogenes* recovered from blood and internal organs at 3 days after inoculation (Fig. 1). This time point was chosen because it typically is the peak of the bacterial burden in the spleen and liver. In contrast, when mice were inoculated intragastrically with *gtcA* mutants of *L. monocytogenes*, we recovered fewer CFU from the spleen, liver, gallbladder, and blood than from mice inoculated with the corresponding parental strain. The parental strains also persisted in greater numbers in the ceca than the corre-

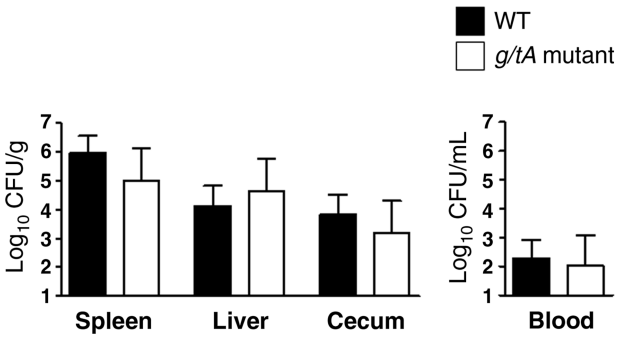

FIG. 1. *gltA* is not required for virulence in intragastrically inoculated mice. Mice were inoculated intragastrically with 10^6 colony-forming units (CFU) of the *gltA* mutant XL7 or the parental strain 4b1 (WT), and bacterial recovery 3 days after inoculation was determined as described in *Materials and Methods*. Results are expressed as the mean ± SEM $\log_{10}$ CFU per mouse (six mice per group).

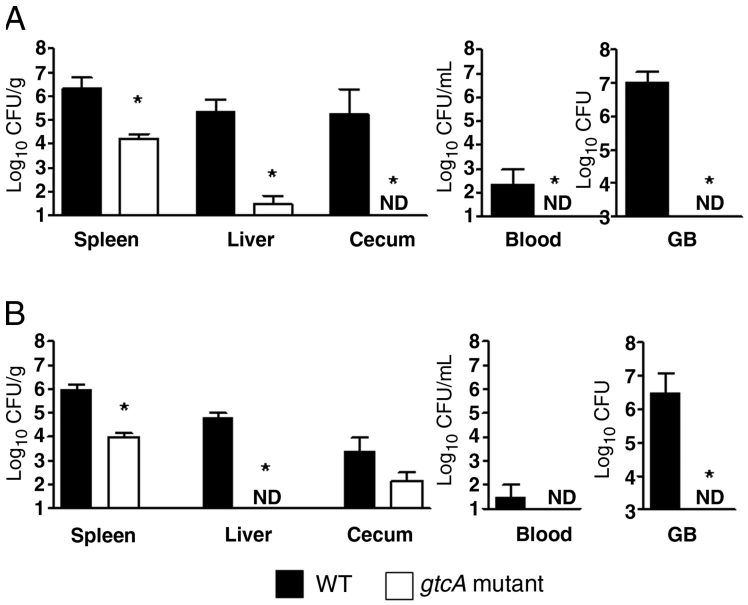

FIG. 2. Impact of *gtcA* mutations on virulence of *L. monocytogenes* serotype 4b in intragastrically inoculated mice. Mice were inoculated intragastrically with 10^6 colony-forming units (CFU) of the *gtcA* mutant 27E8 and its respective parental strain 2381L (**A**) as well as with mutant M44 and its parental strain 4b1 (**B**). Bacterial recovery three days after inoculation was determined as described in *Materials and Methods*. Results are expressed as the mean ± SEM $\log_{10}$ CFU per mouse (six mice per group). GB, gall bladder; ND, not detected (no colonies were recovered from the tissues sampled). * indicates $p < 0.05$ in comparisons between mutant and parental strains.

sponding *gtcA* mutants. We observed similar results with the *gtcA* transposon mutant (strain 27E8) of the outbreak-associated strain 2381L (Fig. 2A), and the *gtcA* mutant (strain M44) of strain 4b1, that was implicated in a sporadic case of listeriosis (Fig. 2B). Both *gtcA* mutants were substantially impaired in their ability to cause systemic infection following IG inoculation of A/J mice. Furthermore, genetic complementation of the *gtcA* mutation partially restored virulence in this murine model. The CFU values of M44pPL2-gtcA recovered from the liver at 3 days after inoculation were significantly greater than those obtained from mice inoculated with strain M44 harboring the integration vector alone (strain M44pPL2) ($p < 0.05$). Although the parental strain 4b1 was recovered at greater numbers than M44pPL2-gtcA, suggesting that restoration of virulence was partial, the differences were not statistically significant ($p > 0.05$) (Fig. 3).

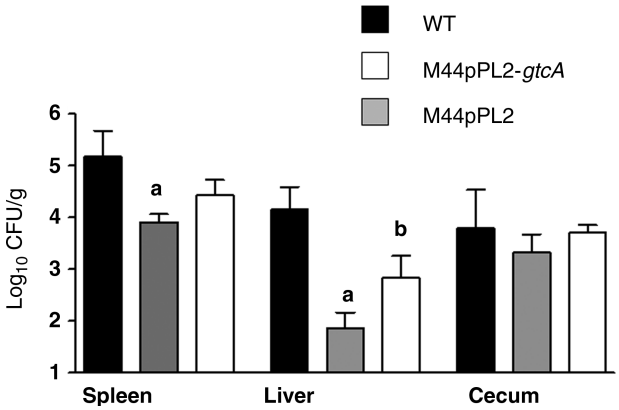

FIG. 3. Complementation of the *gtcA* mutation partially restores virulence in intragastrically inoculated mice. Mice were inoculated intragastrically with 10^6 colony-forming units (CFU) of the *gtcA* mutant M44 containing the empty vector (M44pPL2), the complemented mutant (M44pPL2-*gtcA*) and the parental strain 4b1 (WT). Bacterial recovery 3 days after inoculation was determined as described in *Materials and Methods*. Results are expressed as the mean ± SEM $\log_{10}$ CFU per gram of tissue (six mice per group). $^{a}p < 0.05$ in comparison with the parental strain; $^{b}p < 0.05$ in comparison with the empty vector (M44pPL2).

Effect of gtcA *on virulence in intravenously inoculated A/J mice*

To determine whether the reduced virulence of the *gtcA* mutants was restricted to IG infection, we also evaluated recovery of the *gtcA* mutants and their parental strain counterparts from the spleen and liver following intravenous infections of mice. The CFU of the *gtcA* mutant 27E8 recovered from the liver were significantly lower than those of the parental strain, whereas recovery of strain 27E8 from the spleen was not affected (Fig. 4A). Similar results were obtained using *L. monocytogenes* strain 4b1 and its *gtcA* mutant M44. There was a substantial reduction in the number of CFU recovered from both the

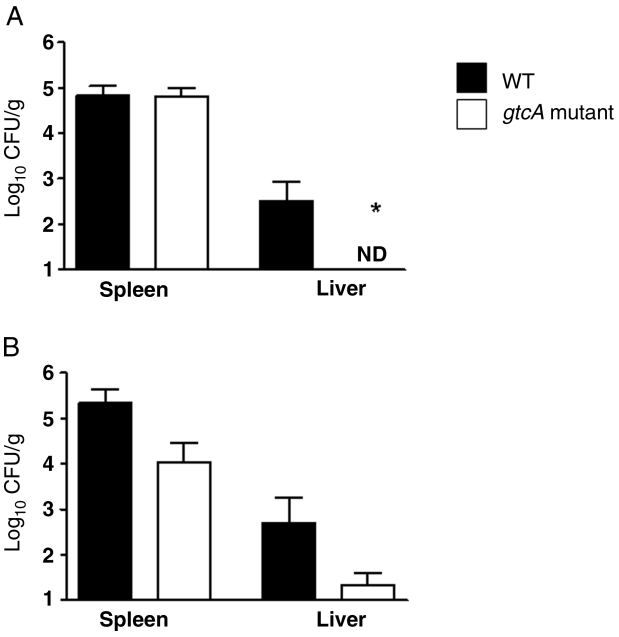

FIG. 4. Impact of *gtcA* mutations on recovery from the liver and spleen following intravenous infections. Mice were inoculated intravenously with 10^3 colony-forming units (CFU) of the *gtcA* mutant 27E8 and its parental strain 2381L (WT) (**A**), or mutant M44 and its parental strain 4b1 (WT) (**B**). Bacterial recovery from liver and spleen 3 days after inoculation was determined as described in *Materials and Methods*. ND, not detected (no colonies were recovered from the tissues sampled). $^*p < 0.05$ for comparisons between mutant and parental strains.

spleens and livers of mice inoculated intravenously with *gtcA* mutant M44 as compared with its parental strain (Fig. 4B).

Invasion of serotype 4b L. monocytogenes *in Caco-2 intestinal epithelial cells requires* gtcA, *but not* gltA

Caco-2 human intestinal epithelial cells were cultured for 18–21 days to allow them to develop the characteristics of mature polarized enterocyte monolayers (i.e., tight junctions, microvilli, and brush border). The *gltA* mutant XL7 was indistinguishable from its parental counterpart in ability to invade and multiply within these differentiated Caco-2 cells (data not shown). Adherence of M44pPL2 (*gtcA* mutant M44 harboring the empty integration vector pPL2) was also indistinguishable from that of the parental strain (Fig. 5A). However, the *gtcA* mutant invaded the differentiated Caco-2 cells to a significantly lesser extent than its parental strain 4b1 (Fig. 5B). The genetically complemented mutant (M44pPL2-gtcA) had partially restored ability to invade Caco-2 cells (Fig. 5B).

When infected Caco-2 cells were incubated for 24 hours *in vitro*, there were fewer CFUs of the *gtcA* mutant than the parental strain 4b1 (Fig. 5C). However, the overall increase in number of CFUs between the time point at which invasion was assessed and 24 hours was similar for the *gtcA* mutant and the parental strain, suggesting that the observed differences reflect differences in invasion, but not in subsequent intracellular multiplication. Impaired invasion of differentiated Caco-2 cells were also

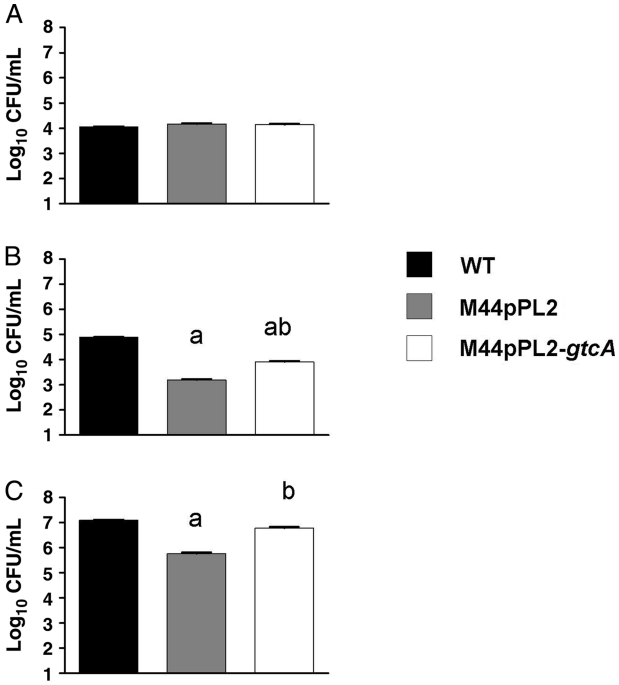

FIG. 5. Impact of *gtcA* mutations on invasion of differentiated Caco-2 cells. Differentiated Caco-2 cells adherent to glass coverslips were incubated with 1×10^7 colony-forming units (CFU) of the parental strain *L. monocytogenes* 4b1 (WT), the *gtcA* mutant M44 containing the empty vector (M44pPL2), or the complemented mutant (M44pPL2-*gtcA*). Attachment (**A**), invasion (**B**), and intracellular growth (**C**) were determined as described in *Materials and Methods*. Data are expressed as the mean $\pm$SEM $\log_{10}$ CFU per well (nine wells per treatment group). Results are from one representative experiment of three such experiments that were performed. $^a p < 0.05$ in comparison with the parental strain; $^b p < 0.05$ in comparison with the empty vector (M44pPL2).

observed for *gtcA* mutant 27E8 as compared to its parental strain (data not shown).

Discussion

It has been demonstrated previously that *gltA* and *gtcA* are required for serotype-specific glycosylation of the teichoic acid of *L. monocytogenes* serotype 4b with galactose and glucose (Promadej *et al*, 1999; Lei *et al*., 2001). Recently we showed that *gtcA*, whose inactivation results in absence of galactose and markedly reduced amounts of glucose on the teichoic acid (Promadej *et al*., 1999), was also required for the ability of serotype 4b bacteria to be infected by both serotype-specific and *Listeria* genus-specific phages (Cheng *et al*., 2008). The results of the present study suggest that *gtcA* is in addition required for gastrointestinal virulence of serotype 4b *L. monocytogenes* in a murine infection model. This was demonstrated by the reduced ability of two different *gtcA* mutants, constructed in different strain backgrounds, to disseminate to blood and internal organs such as liver and spleen following IG inoculation. In contrast, *gltA* (whose inactivation results in absence of glucose but not galactose from the teichoic acid) was not required for gastrointestinal virulence in this animal model. These findings suggest that galactose (but not glucose) on the teichoic acid of serotype 4b bacteria is required for gastrointestinal virulence of *L. monocytogenes* of this serotype in mice. It remains to be determined whether other listerial surface polymers, such as lipoteichoic acid, are also involved in gastrointestinal virulence. A defect in *dltA*, required for D-alanyl-lipoteichoic acid biosynthesis, reduced virulence of *L. monocytogenes* strain L028 (serotype 1/2c) in intravenously inoculated mice (Abachin *et al*., 2002), but impact on virulence in animals inoculated orally or intragastrically was not assessed. Lipoteichoic acid amounts or composition were not affected in the *gtcA* mutants investigated in the current study (Promadej *et al*., 1999).

In the present study we anesthetized mice with sodium pentobarbital before bacterial inoculation, to potentiate the severity of the resulting infection. As described previously we do not know by what mechanism pentobarbital potentiates the severity of infection (Czuprynski *et al*., 2003b). Previous results suggest that it does not reflect alteration in gastric acidity or intestinal motility alone, although we cannot exclude the possibility that these physiological alterations contribute in part to the increased susceptibility to listeriosis (Czuprynski *et al*., 2003b). Previous studies using transgenic mice expressing a functional receptor for internalin A revealed that this determinant is important for virulence of *L. monocytogenes* following oral infections (Lecuit *et al*., 2001). In the murine model employed in the current study, bacterial dissemination past the site of the IG infection involves determinants other than internalin A, since mice do not express a functional receptor for internalin A. We considered such a model desirable, because it was logistically simpler, used a relatively low number of CFU in the inoculum, and allowed evaluation of *gtcA* without possible interference by internalin A. Internalin A expression and control in serotype 4b strains remains poorly characterized, most studies having used bacteria of serotype 1/2a or 1/2c (Cabanes *et al*., 2004; Gaillard *et al*., 1991; Lecuit *et al*., 2001; Milohanic *et al*., 2001; Vazquez-Boland *et al*., 2001; Jacquet *et al*., 2004). It is our intent in the future to investigate virulence of the *gtcA* mutants in an animal model (e.g., guinea pig) expressing a functional internalin A receptor. Such further studies will allow us to determine whether *gtcA* is required for virulence following peroral infections, even in the presence of internalin A–mediated enterocyte invasion.

The requirement for *gtcA* for virulence observed in the current study may be due to the diminished ability of the *gtcA* mutants to invade intestinal epithelial cells. Evidence obtained with Caco-2 human intestinal epithelial cells suggested that the *gtcA* mutants were impaired in their ability to invade these cells. Although the CFU of *L. monocytogenes* recovered after a 24-hour incubation of infected Caco-2 cells was lower for the *gtcA* mutants, the overall increase in CFU was similar for the *gtcA* mutant and the parental strains (approximately $3 \log_{10}$ CFU for both). This finding suggests that the diminished number of CFU at 24 hours reflected impaired invasion, rather than a defect in intracellular multiplication. Genetic complementation of the

gtcA mutation partially restored virulence for mice as well as the ability of the bacteria to invade Caco-2 cells. The reasons for lack of complete complementation are unknown, but may involve differences in expression of the ectopic *gtcA* in the complemented strain in comparison to the parental strain. Partial phenotypic restoration was also observed in another study, which evaluated the ability of phages A500 and A511 to adsorb to 4b1, M44, and M44pPL2-gtcA (Cheng *et al*., 2008). The effect of *gtcA* may extend to other cell types involved in the pathogenesis of listeriosis. In experiments not described in this manuscript, we also observed reduced invasion of murine hepatocytes *in vitro*, and less ability to activate human umbilical vein endothelial cells by *gtcA* mutants of *L. monocytogenes* 4b (data not shown). Thus, we speculate that the ability to decorate teichoic acid with galactose residues is important for the ability of serotype 4b *L. monocytogenes* to interact with several cell types relevant to the pathogenesis of listeriosis.

At this time it is not clear whether the observed impact of *gtcA* in virulence and invasion is specific to serotype 4b strains. In *L. monocytogenes* serotype 4b, *gtcA* is required for serotype specific glycosylation and for reactivity with monoclonal antibodies specific for the serotype 4b complex (Promadej *et al*., 1999). It would be interesting to investigate the role of *gtcA* in the related serotype 4e and 4d strains of *L. monocytogenes*, but those experiments are beyond the scope of the present study. Notable sequence diversity exists between the *gtcA* alleles harbored by strains of serogroup 4b vs. 1 /2 (Autret *et al*., 2001; Glaser *et al*., 2001; Nelson *et al*., 2004; Broad Institute *Listeria monocytogenes* genomic sequence database http://www.broad.mit.edu/annotation/genome/listeria_group/MultiHome.html). Earlier investigations using signature-tagged mutagenesis and a murine parenteral infection model revealed that *gtcA* mutants of the serotype 1/2a strain EGD-e were attenuated in their ability to disseminate to and multiply in the brains of intravenously inoculated mice (Autret *et al*., 2001). Because serotype 1/2a *L. monocytogenes* lack galactose residues on their teichoic acid (instead harboring rhamnose as serogroup-specific teichoic acid substituent) (Uchikawa *et al*., 1986), we presume that *gtcA* plays an as yet to be determined role, other than decorating teichoic acid with galactose, in this serotype. It would be of great interest to determine whether *gtcA* is required for virulence of strain EGD-e and other serogroup 1/2 strains following IG inoculation.

Recently, the gene lmo2537, encoding a precursor of the teichoic acid linkage unit, was shown to be essential for survival and growth of *L. monocytogenes* EGD-e (Dubail *et al*., 2006), and it is likely that teichoic acid is an essential structural component in *L. monocytogenes* of other serotypes as well. It is tempting to speculate that differences in glycosylation of this indispensable cell wall–associated polymer, mediated by serotype-specific *gtcA* alleles, are involved in virulence of *L. monocytogenes*. Future studies will elucidate the mechanism by which serotype-specific glycosylation of the teichoic acid impact listerial pathogenesis in the gastrointestinal tract, and perhaps at other relevant sites such as the brain or placenta.

Conclusions

The results of this study show that inactivation of *gtcA*, required for serotype-specific glycosylation of the teichoic acid of *L. monocytogenes* serotype 4b with galactose and glucose, also impaired virulence of *L. monocytogenes* in mice inoculated via gastric gavage, and reduced invasion into Caco-2 human intestinal epithelial cells. These findings suggest that *gtcA* is an important contributor to virulence of serotype 4b *L. monocytogenes*, the serotype that is associated with most large outbreaks of foodborne listeriosis.

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Disclosure Statement

No competing financial interests exist.

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